



**EASTERN REGIONAL RESEARCH CENTER
AGRICULTURAL RESEARCH SERVICE
UNITED STATES DEPARTMENT OF AGRICULTURE
600 E. MERMAID LANE
WYNDMOOR, PA 19038
(215) 233-6400**

Title: Scanning Electron Microscopy of Native Biofilms on Mung Bean Sprouts

Author(s): W.F. Fett and P.H. Cooke

Citation: Canada Journal of Microbiology (2003) 49: 45-50

Number: 7213

Please Note:

This article was written and prepared by U.S. Government employees on official time, and is therefore in the public domain.

Our on-line publications are scanned and captured using Adobe Acrobat. During the capture process some errors may occur. Please contact William Damert, wdamert@arserrc.gov if you notice any errors in this publication.

Scanning electron microscopy of native biofilms on mung bean sprouts¹

William F. Fett and Peter H. Cooke

Abstract: Native biofilms present on the adaxial surface of cotyledons of mung bean sprouts (*Vigna radiata*) were studied by use of scanning electron microscopy. Biofilms were abundant on the cotyledon surfaces and were comprised of rod-shaped bacteria, cocci-shaped bacteria, or yeasts, often with one type of microbe predominant. In contrast to our earlier study of biofilms on green sprouts (alfalfa, clover, broccoli, and sunflower), yeast and cocci were abundant on mung bean. Filamentous fungi were not observed. Sheet-like or fibrillar material (presumably composed of secreted microbial polysaccharides, proteins, lipids, and nucleic acids) fully or partially covered the biofilms. Biofilms up to 5 mm in length were observed, and some biofilms were comprised of more than just a monolayer of microbial cells. Native biofilms on sprout surfaces undoubtedly play an important role in the ecology of plant epiphytic microbes and may also afford protected sites for plant and human bacterial pathogens.

Key words: mung bean sprouts, biofilms, native microflora, scanning electron microscopy, food safety.

Résumé : Nous avons examiné par microscopie électronique à balayage des biofilms natifs présents sur la surface adaxiale de cotylédons de pousses de haricots mungo (*Vigna radiata*). Les biofilms étaient abondants sur les surfaces des cotylédons et étaient composés de bactéries en forme de bâtonnets, de cocques ou de levures. Un type de microbe prédominait fréquemment. Contrairement à notre étude précédente sur les biofilms présents à la surface de pousses vertes (luzerne, trèfle, brocoli et tournesol), les levures et les cocques étaient abondantes sur les haricots mungo. Nous n'avons pas observé de champignons filamenteux. De la matière en feuillets ou fibreuse (vraisemblablement composée de polysaccharides sécrétés, de protéines, de lipides et d'acides nucléiques d'origine microbienne) recouvraient partiellement ou totalement les biofilms. Les biofilms de longueur allant jusqu'à 5 mm ont été observés et certains d'entre eux étaient formés de plus d'une monocouche de cellules microbiennes. Les biofilms natifs qu'on retrouve à la surface de pousses jouent sans aucune doute un rôle important dans l'écologie des microbes épiphytiques des plantes et pourraient également fournir des sites privilégiés pour des bactéries causant des maladies chez les végétaux ou chez l'humain.

Mots clés : pousses de haricots mungo, biofilms, microflore indigène, microscopie électronique à balayage, sûreté des aliments.

[Traduit par la Rédaction]

Introduction

During the 1990's, recorded outbreaks of foodborne illness in the U.S.A. due to the consumption of alfalfa (*Medicago sativa*) and clover (*Trifolium* spp.) sprouts contaminated with *Salmonella* spp. or *Escherichia coli* O157 increased dramatically (Taormina et al. 1999; NACMCF 1999). Since 1988, several foodborne outbreaks due to mung bean sprouts (*Vigna radiata*) contaminated with *Salmonella* spp. have occurred in

several countries, including the United Kingdom, the U.S.A., the Netherlands, and Canada (Anonymous 2000; Honish and Nguyen 2001; O'Mahony et al. 1990). The primary source of bacterial human pathogens appears to be the sprouting seed based on epidemiological evidence as well as, in some instances, direct isolation from implicated seed lots (NACMCF 1999; Taormina et al. 1999). Sprouts are considered to be a special food safety problem, because populations of bacterial pathogens are capable of increasing from low lev-

Received 12 August 2002. Revision received 25 November 2002. Accepted 18 December 2002. Published on the NRC Research Press Web site at <http://cjm.nrc.ca> on 7 February 2003.

W.F. Fett¹ and P.H. Cooke. Food Safety Intervention Technologies Research Unit, Eastern Regional Research Center, Agricultural Research Service (ARS), USDA, Wyndmoor, PA 19038, U.S.A.

¹Mention of brand or firm name does not constitute an endorsement by the U.S. Department of Agriculture above others of a similar nature not mentioned.

²Corresponding author (e-mail: wfett@arserrc.gov).

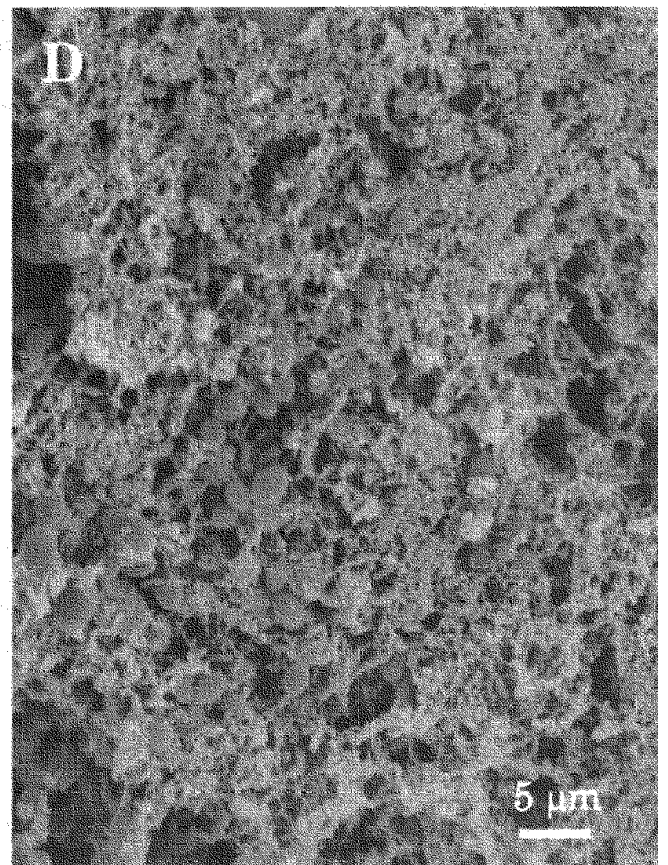
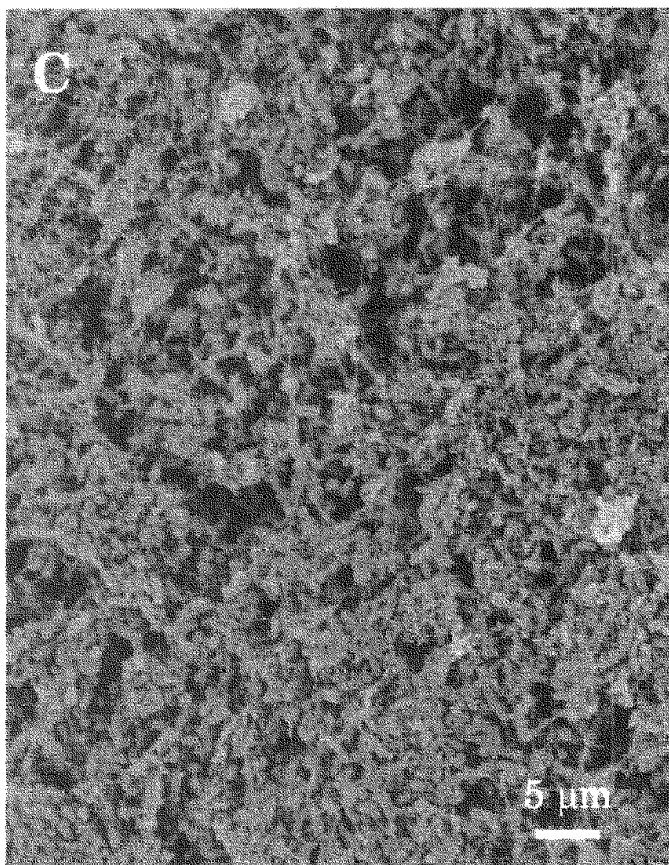
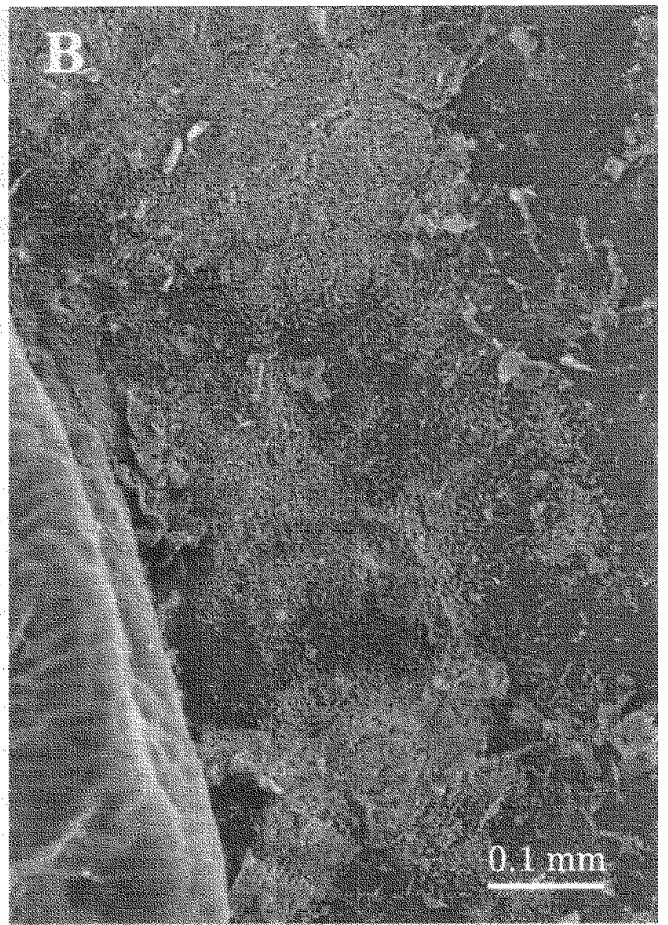
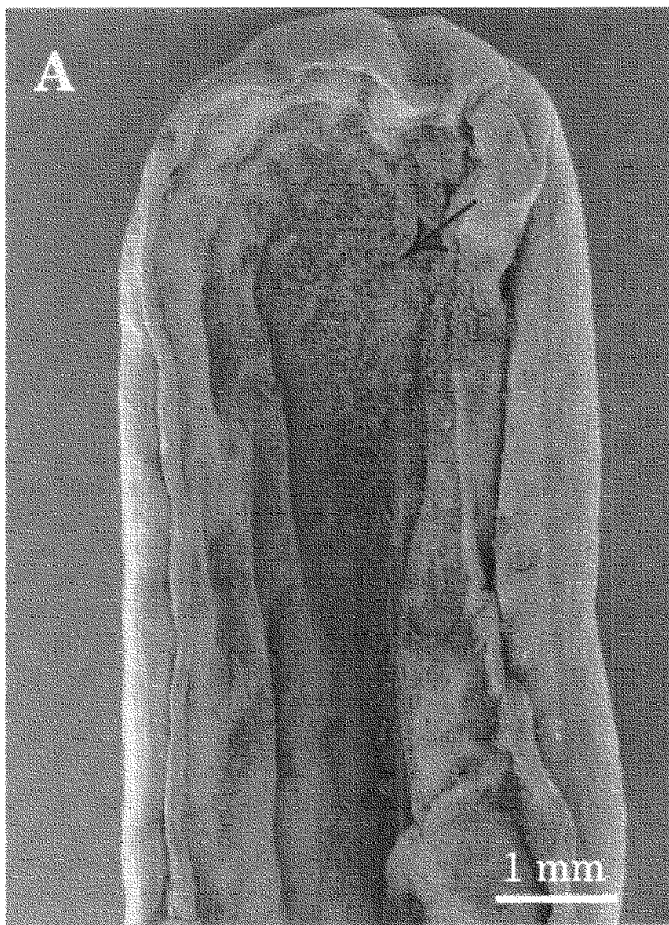


Fig. 1. Scanning electron micrographs of native biofilms on mung bean (*Vigna radiata*) cotyledons (adaxial side). (A) Rugose surface of a cotyledon showing extensive amorphous material on the surface (arrow). (B) Higher magnification showing an area of biofilm next to a fold in the cotyledon surface. (C) High magnification of a biofilm consisting of densely packed bacterial cells covered by fibrillar material. (D) High magnification of a biofilm consisting of densely packed yeast covered by fibrillar material.

els on seed to high levels (up to 7 log CFU/g) during the growth of sprouts (Jaquette et al. 1996; Stewart et al. 2001a, 2001b; Taormina and Beuchat 1999). In addition, alfalfa and clover sprouts are consumed raw, without a kill step between harvest and consumption (NACMCF 1999). Mung bean sprouts are consumed both lightly cooked and raw.

In the natural environment, as well as industrial and medical settings, bacteria are often present in biofilms (Costerton et al. 1995; Davey and O'Toole 2000). Biofilms are assemblages of microbes attached to a surface and to each other by a matrix composed of exopolysaccharides and other excreted materials of microbial origin. Biofilms can range in structure, occurring as monolayers to macroscopic communities, and in natural environments, they usually consist of multiple microbial species (O'Toole et al. 2000; Wimpenny et al. 2000). Mature biofilms can be highly heterogeneous, composed of microcolonies (aggregates) of microbes enclosed in bacterial exopolysaccharide and separated by water channels (Costerton et al. 1995).

Microbial adherence and subsequent biofilm formation are of interest to the food science as well as microbial ecology fields. Pathogenic as well as spoilage microorganisms present in biofilms are much more difficult to remove physically from foods and processing equipment, and bacteria in biofilms may be more resistant to the effects of antimicrobial agents, up to 500-fold or greater when compared with their free-living counterparts (Bower et al. 1996; Costerton et al. 1995; Frank 2001; Kumar and Anand 1998). Introduced bacterial human pathogens may be able to form homogeneous biofilms on sprouts but also may become part of biofilms composed of the native microflora.

In contrast with biofilms present on nonliving surfaces, the study of biofilms on the surface of plants including fruits and vegetables is in its infancy. A previous study in our laboratory using scanning electron microscopy (SEM) indicated that native biofilms are abundant on the surfaces of a variety of so-called "green" (chlorophyll-containing) sprouts (alfalfa, clover, broccoli (*Brassica oleracea*), and sunflower (*Helianthus annuus*)) (Fett 2000). Biofilms were most easily observed on cotyledon surfaces as compared with hypocotyls and roots, and biofilms covering almost entire cotyledons (up to 2 mm in length) were observed. On these sprout types, biofilms composed solely of rod-shaped bacteria were predominant. Cocci-shaped bacteria and yeasts were rarely seen, and in no instances were filamentous fungi present as members of the biofilms on the sprouts. Differing from native biofilms observed on roots by SEM, biofilms that were observed on cotyledons and hypocotyls appeared to be composed of monolayers of bacterial cells.

In commercial operations, most green sprouts including alfalfa and clover are grown hydroponically in rotating drums or trays and are exposed to light. In contrast, mung bean sprouts are grown hydroponically in bins kept in the dark. In this study, we sought to determine (i) if biofilms are present on mung bean sprouts, a non-green sprout type, (ii) the com-

position of the biofilms (bacteria, yeasts, and (or) higher fungi), and (iii) if the biofilms are comprised of more than just a monolayer of microbes.

Source of mung bean sprouts

Mung bean sprouts were purchased from two retail outlets and were from two separate suppliers. One sample was displayed in a refrigerated, open-faced display case and was packaged in a clam shell plastic container. The second sample was from unpackaged mung bean sprouts sold in bulk in an open bin without refrigeration in an ethnic supermarket. Only the first sample had a "sell by" date, and this sample of sprouts was purchased one day prior to this date. Sprouts that appeared fully turgid and were free from visible fungal and bacterial rots were selected.

Scanning electron microscopy

On the day of purchase, cotyledons were transected, using fine scissors, at the junction with the hypocotyls (eight pairs from each source), immersed in 20 mL of 2.5% glutaraldehyde prepared in 0.1 M imidazole-HCl buffer, pH 7.0, and stored at 4°C for 3 days. The pairs of cotyledons were then separated by cutting with a stainless steel razor blade, washed twice with buffer alone, immersed in 2% osmium tetroxide prepared in buffer for 3 h, washed with distilled water, and sequentially dehydrated in a graded series of ethanol (50%, 80%, and absolute). The cotyledons were critical-point-dried from liquid carbon dioxide, and the dried samples were mounted on aluminum specimen stubs with colloidal silver adhesive paste (Electron Microscopy Sciences, Fort Washington, Pa.). Finally, the mounted samples were coated with a thin layer of gold using a sputter apparatus (Edwards High Vacuum, Wilmington, Mass.), and the adaxial surfaces were examined. Digital images were collected using an Imix-1 digital imaging workstation (Princeton Gamma-Tech., Princeton, N.J.) integrated with a model JSM840A scanning electron microscope (JEOL U.S.A., Peabody, Mass.) operated in the secondary electron imaging mode at 10 kV. Samples were surveyed and imaged at $\times 15$, $\times 150$, $\times 1500$, and $\times 5000$.

Biofilms on mung bean sprout cotyledons

After fixation and drying, the adaxial cotyledon surfaces were concave, ranging from 7 to 9 mm in length and from 3 to 4 mm in width (Fig. 1A). The adaxial surfaces were either smooth or rugose with a few longitudinally oriented ridges and folds, creating valleys and crevices that were frequently about 1–2 mm below the upper edges (Fig. 1A). At low magnification, some areas on the adaxial surfaces were differentiated by the outlines of epidermal cells and cell junctions (not shown), while these features for other areas were obscured by the presence of extensive amorphous material

Fig. 2. Scanning electron micrographs of native biofilms on mung bean (*Vigna radiata*) cotyledons (adaxial side). (A) Biofilm composed of cocci-shaped bacteria, which are partially covered by a sheet-like material (arrow). (B) Biofilm composed of rod-shaped bacteria, which are partially covered by a sheet-like material (arrow). (C) Dense biofilm extending away from the plant surface. (D) Very high magnification of heterogenous bacteria in a biofilm. Note surface fibrils and blebs (arrows).

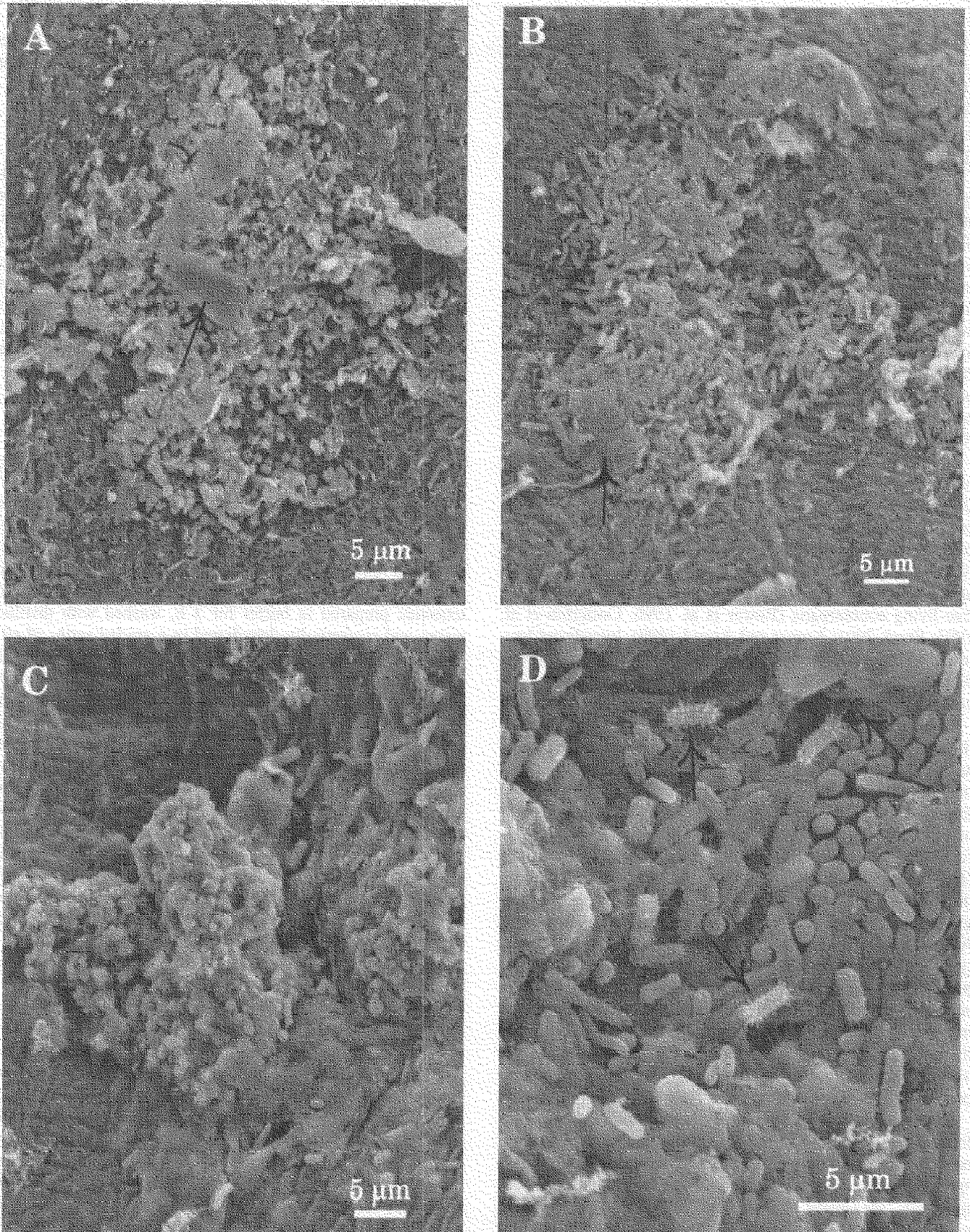
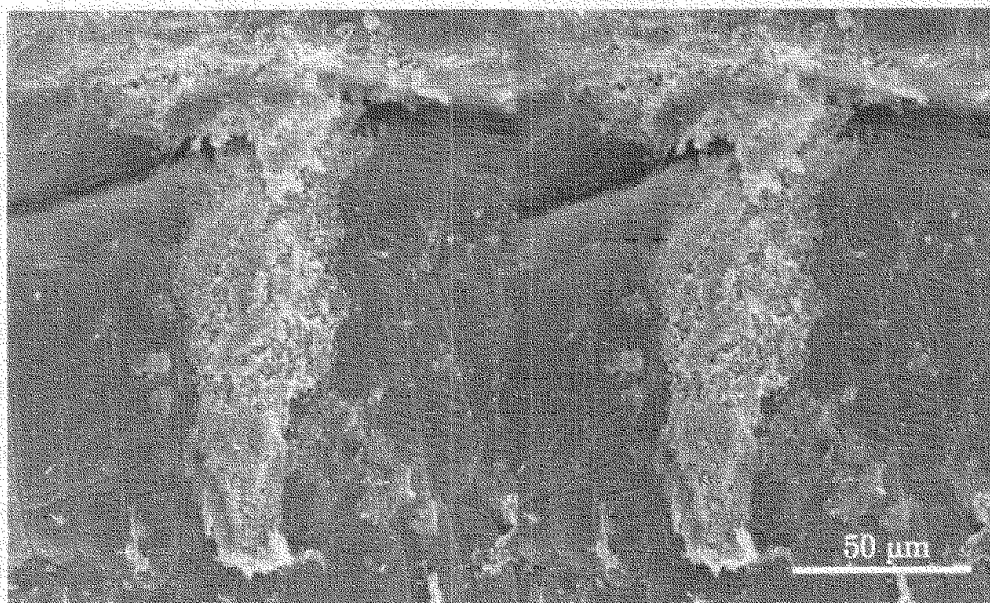


Fig. 3. Scanning electron micrograph (stereo image) of a multilayered native biofilm on a mung bean (*Vigna radiata*) cotyledon (adaxial side). The image is best viewed with a pocket or lorgnette stereo viewer.



(Fig. 1A). Upon examination at higher magnification, the amorphous material was found to consist of microbial biofilms (Figs. 1B, 1C, 1D) that were up to 5 mm in length (Fig. 1A). The composition of the biofilms was variable. Most were made up of bacterial cells (Fig. 1C), sometimes with a low number of yeast cells also present (not shown). Occasionally, biofilms primarily composed of yeast were observed (Fig. 1D). Microorganisms were identified as yeasts based on their size, bulbous morphology, and evidence of division by budding. Some biofilms were composed of cocci-shaped bacteria (Fig. 2A), others of rod-shaped bacteria (Fig. 2B), and some were a mixture of cocci and rods (Fig. 2D). Microbes in biofilms were often covered (ranging from partially to almost totally) with either fibrillar (Figs. 1C and 1D) or sheet-like material (Figs. 2A and 2B). At the highest magnification employed, fibrils were often seen attaching bacteria to the plant surface and to each other, and some bacterial cells exhibited blebs on their surfaces (Fig. 2D). Biofilms were observed that consisted of a few microbial layers (Figs. 1C, 1D, 2A, and 2B), as well as many layers (Figs. 2C and 3).

Discussion

Our previous SEM study of alfalfa, broccoli, clover, and sunflower sprouts indicated that bacterial biofilms were present on all plant parts (roots, hypocotyls, and cotyledons) but were most abundant on cotyledons (Fett 2000). In that study, except for some biofilms present on roots, there was no evidence that biofilms consisted of more than a single layer of bacterial cells. The results of the current SEM study of mung bean cotyledons differed from our previous study of green sprout cotyledons in several ways. First, cocci-shaped bacterial cells as well as yeast were much more abundant on the mung bean cotyledons, and both were found to form biofilms. Also, micrographs indicated that biofilms on mung bean cotyledons were often more than a single bac-

terial layer in depth. Additional studies with larger and more diverse samples are required to confirm these differences. Yeast are capable of initiating biofilms on inert surfaces (Reynolds and Fink 2001) and, based on our study, appear to be able to initiate biofilms on cotyledon surfaces as well.

The sheet-like and fibrillar material that fully or partially coated microbes present in biofilms, as well as bacterial cell surface fibrils and blebs, were most likely highly hydrated (in their natural state) bacterial exopolysaccharides and other excreted bacterial extracellular materials (proteins, nucleic acids, and lipids) that had dried during the processing of the samples for microscopy (Costerton et al. 1995; Davey and O'Toole 2000; Sutherland 2001; Whitchurch et al. 2002). Previously, biofilms covered by sheet-like material were observed on plant leaves by SEM (Bjorklof et al. 2000; Morris et al. 1997). These biofilms consisted of either native microflora or a pseudomonad that was used for inoculation. The extracellular matrix is believed to protect bacteria from desiccation on plant surfaces (Romanschuk et al. 1996).

Studies employing SEM indicate that native biofilms are present on a variety of plant surfaces (Fett 2000; Fuqua and Matthyse 2001; Itoh et al. 2001; Morris et al. 1997; Sharga 1997; Sutherland 1996). Recently, native biofilms present on leafy vegetables have been studied using confocal scanning laser microscopy (CSLM) (Carmichael et al. 1999; Morris et al. 1997). CSLM does not require fixation or drying of samples. Studies by Morris et al. (1997, 1998) using CSLM indicated that biofilms on field-grown leafy vegetables are up to 20 μm in depth and 1 mm in length and are heterogeneous in nature, composed of bacteria, filamentous fungi, and yeasts.

The presence of native biofilms on mung bean sprouts affords microbes that are pathogenic to plants or humans potential colonization sites and protection from both physical removal and killing by antimicrobials. CSLM is currently being employed in our laboratory to further characterize native biofilms on sprouts.

Acknowledgements

We thank Mr. Paul Pierlott for assistance in preparation of the micrographs for publication.

References

- Anonymous. 2000. Salmonellosis outbreak associated with raw mung bean sprouts [online]. Press Release 19 April 2000. Available from <http://www.dhs.ca.gov> [cited 28 January 2003].
- Bjorklof, K., Nurmiaho-Lassila, E.-L., Klinger, N., Hahtela, K., and Romantschuk, M. 2000. Colonization strategies and conjugal gene transfer of inoculated *Pseudomonas syringae* on the leaf surface. *J. Appl. Microbiol.* **89**: 423–432.
- Bower, C.K., McGuire, J., and Daeschel, M.A. 1996. The adhesion and detachment of bacteria and spores on food contact surfaces. *Trends Food Sci. Technol.* **7**: 152–157.
- Carmichael, I., Harper, I.S., Coventry, M.J., Taylor, P.W.J., Wan, J., and Hickey, M.W. 1999. Bacterial colonization and biofilm development on minimally processed vegetables. *J. Appl. Microbiol. Symp. Suppl.* **85**: 45S–51S.
- Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R., and Lappin-Scott, H.M. 1995. Microbial biofilms. *Annu. Rev. Microbiol.* **49**: 711–745.
- Davey, M.E., and O'Toole, G.A. 2000. Microbial biofilms: from ecology to molecular genetics. *Microbiol. Mol. Biol. Rev.* **64**: 847–867.
- Fett, W.F. 2000. Naturally occurring biofilms on alfalfa and other types of sprouts. *J. Food Prot.* **63**: 625–632.
- Frank, J.F. 2001. Microbial attachment to food and food contact surfaces. *Adv. Food Nutr. Res.* **43**: 319–369.
- Fuqua, C., and Matthisse, A.G. 2001. Methods for studying bacterial biofilms associated with plants. *Methods Enzymol.* **337**: 3–18.
- Honish, L., and Nguyen, Q. 2001. Outbreak of *Salmonella enteritidis* phage type 913 gastroenteritis associated with mung bean sprouts – Edmonton, 2001. *Can. Comm. Dis. Rep.* **27**(18): 151–156.
- Itoh, S., Yoshida, K., Isobe, S., and Itoh, K. 2001. Decontamination of lettuce using acidic electrolyzed water. *J. Food Prot.* **64**: 652–658.
- Jaquette, C.B., Beuchat, L.R., and Mahon, B.E. 1996. Efficacy of chlorine and heat treatment in killing *Salmonella* Stanley inoculated onto alfalfa seeds and growth and survival of the pathogen during sprouting and storage. *Appl. Environ. Microbiol.* **62**: 2212–2215.
- Kumar, C.G., and Anand, S.K. 1998. Significance of microbial biofilms in food industry: a review. *Int. J. Food Microbiol.* **42**: 9–27.
- Morris, C.E., Monier, J.-M., and Jacques, M.-A. 1997. Methods for observing microbial biofilms directly on leaf surfaces and recovering them for isolation of culturable microorganisms. *Appl. Environ. Microbiol.* **63**: 1570–1576.
- Morris, C.E., Monier, J.-M., and Jacques, M.-A. 1998. A technique to quantify the population size and composition of the biofilm component in communities of bacteria in the phyllosphere. *Appl. Environ. Microbiol.* **64**: 4789–4795.
- National Advisory Committee on Microbiological Criteria for Foods. 1999. Microbiological safety evaluations and recommendations on sprouted seeds. *Int. J. Food Microbiol.* **52**: 123–153.
- O'Mahony, M., Cowden, J., Smyth, B., Lynch, D., Hall, M., Rowe, B., Teare, E.L., Tettmar, R.E., Rampling, A.M., Coles, M., Gilbert, R.J., Kingcott, E., and Bartlett, C.L.R. 1990. An outbreak of *Salmonella saint-paul* infection associated with beansprouts. *Epidemiol. Infect.* **104**: 229–235.
- O'Toole, G., Kaplan, H.B., and Kolter, R. 2000. Biofilm formation as microbial development. *Annu. Rev. Microbiol.* **54**: 49–79.
- Reynolds, T.B., and Fink, G.R. 2001. Baker's yeast, a model for fungal biofilm formation. *Science (Washington, D.C.)*, **291**: 878–881.
- Romantschuk, M., Roine, E., Bjorklof, K., Ojanen, T., Nurmiaho-Lassila, E.-L., and Hahtela, K. 1996. Microbial attachment to plant aerial surfaces. In *Aerial plant surface microbiology*. Edited by C.E. Morris, P.C. Nicot, and C. Nguyen-The. Plenum Publishing Corporation, New York. pp. 43–57.
- Sharga, B.M. 1997. *Bacillus* isolates as potential biocontrol agents against chocolate spot on Faba beans. *Can. J. Microbiol.* **43**: 915–924.
- Stewart, D.S., Reineke, K., Ulaszek, J., Fu, T., and Tortorello, M. 2001a. Growth of *Escherichia coli* O157:H7 during sprouting of alfalfa seed. *Lett. Appl. Microbiol.* **33**: 95–99.
- Stewart, D.S., Reineke, K.F., Ulaszek, J.M., and Tortorello, M.L. 2001b. Growth of *Salmonella* during sprouting of alfalfa seeds associated with salmonellosis outbreaks. *J. Food Prot.* **64**: 618–622.
- Sutherland, I.W. 1996. A natural terrestrial biofilm. *J. Ind. Microbiol.* **17**: 281–283.
- Sutherland, I.W. 2001. Biofilm exopolysaccharides: a strong and sticky framework. *Microbiology (Reading, U.K.)*, **147**: 3–9.
- Taormina, P.J., and Beuchat, L.R. 1999. Behavior of enterohemorrhagic *Escherichia coli* O157:H7 on alfalfa sprouts during the sprouting process as influenced by treatments with various chemicals. *J. Food Prot.* **62**: 850–856.
- Taormina, P.J., Beuchat, L.R., and Slutsker, L. 1999. Infections associated with eating seed sprouts: an international concern. *Emerg. Infect. Dis.* **5**: 626–634.
- Whitchurch, C.B., Tolker-Nielsen, T., Ragas, P.C., and Mattick, J.S. 2002. Extracellular DNA required for bacterial biofilm formation. *Science (Washington, D.C.)*, **295**: 1487.
- Wimpenny, J., Manz, W., and Szwedzyk, U. 2000. Heterogeneity in biofilms. *FEMS Microbiol. Rev.* **24**: 661–671.